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Resistance to pH decline and slower calpain-1 autolysis are associated with higher energy availability early *postmortem* in *Bos taurus indicus* cattle

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ABSTRACT

Beef from *Bos taurus indicus* is associated with toughness compared to *Bos taurus taurus*, suggesting there is antagonism between adaptability to heat and beef quality. Resistance to cellular stress in muscle may be protective *postmortem*, thereby delaying its conversion to meat. Therefore, our objective was to determine pH decline, calpain-1 and caspase 3 activation, and proteolysis in different biological cattle types. Angus, Brangus, and Brahman steers (n = 18) were harvested, and *Longissimus lumborum* were assessed *postmortem* for pH decline, ATP content, protease activation, and calpastatin content; and myofibrillar protein degradation was evaluated in beef aged to 14d. Brahman *Longissimus lumborum* exhibited resistance to pH decline, greater ATP content at 1 h, and delayed calpain-1 autolysis. Although content of caspase-3 zymogen was lower in Brahman, there was no evidence of caspase-3 mediated proteolysis. Greater resistance to energetic and pH changes early *postmortem* in Brahman *Longissimus lumborum* are associated with calpain-1 autolysis but not mitochondria mediated apoptosis.

1. Introduction

Cattle biological types vary regarding their performance and meat quality. Compared to taurine, indicine cattle produce less tender beef after the same aging period (Crouse, Cundiff, Koch, Koohmaraie, & Seideman, 1989; Johnson, Huffman, Williams, & Hargrove, 1990; Koch, Dikeman, & Crouse, 1982; Wheeler, Shackelford, Koohmaraie, Koch, & Crouse, 1996). Tenderness differences between subspecies are primarily attributed to increased calpastatin: calpain activity in indicine beef, which results in reduced breakdown of muscle proteins during aging (Pringle, Harrelson, West, Williams, & Johnson, 1999; Pringle, Williams, Lamb, Johnson, & West, 1997; Wheeler, Savel, & Cross, 1990; Whipple et al., 1990). Calpain-1 is generally considered the main protease involved in *postmortem* breakdown of muscle proteins, whereas calpastatin is the only known inhibitor specific for calpains. Although the greater calpastatin: calpain activity of indicine muscle is well-documented, the biological basis and its relationship to heat tolerance in the living animal is poorly understood. It is anticipated that adaptations in *Bos taurus indicus* muscle promote cell survival and improve cellular resistance to stress. Accordingly, these mechanisms persist in *postmortem* muscle, thereby protecting the cell against death and ultimately delaying tenderization.

Maintaining energy (ATP) levels in muscle and stabilizing cell structures would be important protective mechanisms. Along these lines, steers with a high proportion of Brahman genetics exhibit higher pH of the *Longissimus lumborum* (LL) at 3 h *postmortem* compared to steers with a high proportion of Angus (Wright et al., 2018). Higher pH is expected to coincide with higher ATP levels (Marsh, 1954). Further, higher pH is more favorable for calcium uptake by sarcoplasmic reticulum and mitochondria (Whiting, 1980), and greater ATP is available to support sarcoplasmic reticulum calcium pumps for re-sequestering calcium. In turn, these conditions would be expected to limit increases in sarcoplasmic calcium and delay calpain activation (reviewed by Goll, Thompson, Li, Wei, & Cong, 2003). Differences in the rate of pH decline indicate that metabolic or functional properties may also differ between groups. Interestingly, LL citrate synthase activity was positively associated with increasing percentage of Brahman composition, suggesting that mitochondrial content may be higher with greater *Bos taurus indicus* influence (Wright et al., 2018).

Mitochondria have been implicated in several aspects of *postmortem* metabolism and meat quality development, including tenderization. During early stages *postmortem,* mitochondria have the potential to delay calpain activation by sequestering calcium. Mitochondria function also affects pH decline; in an in vitro system modeling *postmortem*

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metabolism, inhibition of mitochondrial enzymes accelerated anaerobic glycolysis and pH decline (Scheffler, Matarneh, England, & Gerrard, 2015). Therefore, mitochondrial function could contribute to different acidification rates between genotypes. Others have suggested that mitochondria-mediated apoptosis enhances proteolysis in *postmortem* skeletal muscle by activating caspases (Cao et al., 2010; Gagaoua, Claudia Terlouw, Boudjellal, & Picard, 2015; Kemp, Bardsley, & Parr, 2006; Ouali et al., 2006). Changes in cellular environment or mitochondrial properties can trigger release of cytochrome *c* from mitochondria to the cytosol, followed by initiation and execution of the death signal by caspases (Liu, Kim, Yang, Jemmerson, & Wang, 1996). The executioner caspase-3 can cleave calpastatin (Wang et al., 1998); this could reduce inhibition of calpain, leading to greater proteolysis and tenderization. Conversely, anti-apoptotic factors such as heat shock proteins (HSPs) can influence the process by controlling caspase-3 activation (Li, Lee, Ko, Kim, & Seo, 2000). Therefore, diverse cattle biological types may be a good model to understand early *postmortem* metabolism and apoptosis related events, and their contribution to beef tenderization.

The objective of the present study was to determine the relationship between pH decline *postmortem,* calpain-1 and caspase-3 activation, and proteolysis between biological types. Additionally, mitochondrial proteins and HSP70 content were investigated. We hypothesized that LL of *Bos taurus indicus* possesses greater resistance to cellular stresses; and in *postmortem* muscle, this manifests as slower pH decline, and delayed calpain-1 autolysis and onset of apoptosis, ultimately resulting in reduced proteolysis.

2. Materials and methods

This study is based on cattle from a long-term genetic evaluation study involving Angus (An), Brahman (Bm), and Angus-Brahman crossbreeds. Six animals from each of the following three breed groups (n = 18): Brahman (100 to 80% Bm and 0 to 20% An); Angus (100 to 80% An and 0 to 20% Bm) and Brangus (Bg; 62.5% An and 37.5% Bm) (Elzo, Johnson, Wasdin, & Driver, 2012) belonging to University of Florida herd were used for the experiment. Three sires are represented in Angus and Brangus groups, while four sires are represented in Brahman group; of these sires, one has offspring in the Brahman and Brangus groups (one per group). Standards for animal care and use were approved by the University of Florida Institutional Animal Care and Use Committee (IACUC number 201503744).

2.1. Animals and sampling

Steers, averaging 18-months of age, were transported approximately 55 km to the University of Florida Meat Processing Center (Gainesville, FL) on the day prior to slaughter. Steers were harvested under USDA-FSIS inspection on three different dates, with two animals from each biological type slaughtered on each day. Samples from *Longissimus lumborum* (LL) muscle were collected at 1, 3, 6 and 24 h *postmortem* and immediately frozen in liquid nitrogen accompanied by pH assessment at 1, 3, 6, 9 and 24 h using a meat pH meter (Hanna HI99163, Hanna Instruments, Woonsocket, RI). The meter was calibrated according to manufacturer and carcass allocation, with re-calibration performed every time that reading of buffer 7 was lower than 6.95 or higher than 7.05. For calibration at cold, the buffers were previously equilibrated to temperature of room. For temperature assessment, a probe was inserted at near position were pH was assessed. At 48 h *postmortem*, carcasses were ribbed between 12 and 13th rib and two steaks were collected and aged for 7 and 14d when samples were collected for immunodetection analysis.

2.2. ATP content

Approximately 100 mg of powdered LL muscle collected at 1, 3 and 6 h *postmortem* was used to determined adenosine triphosphate (ATP) content as previously described by Copenhafer, Richert, Schinckel, Grant, and Gerrard (2006), with concentration reported as μmol ATP/g tissue.

2.3. Enzymatic activities

Using samples collected 1 h *postmortem*, citrate synthase (CS) and lactate dehydrogenase (LDH) activities were determined according to Wright et al. (2018). Activity was calculated using Beer-Lambert equation and are reported as nmol/min/mg tissue for CS and μmol/min/mg tissue for LDH.

2.4. Sample extraction for SDS-PAGE and immunodetection analysis

For analysis of mitochondrial proteins (ATP synthase subunit alpha–ATP5A and cytochrome *c* oxidase subunit 4–COX4), 50 mg powdered LL samples (1 h *postmortem*) were diluted in 500 μl extraction buffer (50 mM Tris-base; 1 mM EDTA, pH adjusted at cold to 7.5 with addition of 10% glycerol, 1% triton X, 50 mM sodium fluoride, 1 mM dithiothreitol and 5 μl/ml protease cocktail inhibitor; P8340, Sigma, St. Louis, MO). Samples were homogenized using a bead-beating homogenizer at 5000 rpm for 10 s (Precellys 24 Homogenizer, Bertin Instruments, Rockville, MD) followed with sonication (10 times/1 s), and incubation on ice for 20 min (Wadley and McConell, 2007). Samples were centrifuged at 10,000 ×*g* for 20 min at 4 °C and supernatant was collected. Protein concentration was determined using Pierce protein assay with ionic detergent compatibility reagent (Thermo Scientific, Rockford, IL). Samples were diluted with Laemmli buffer (final concentration: 40 mM Tris, pH 6.8; 100 mM dithiothreitol, 2% SDS, 0.01% bromophenol blue, 10% glycerol) to yield equal protein concentrations; then samples were heated at 95 °C for 5 min and kept at −20 °C until loaded in gels.

Calpastatin was extracted from powdered LL samples (1 h, 24 h, and 14d *postmortem*) using cold extraction buffer (100 mM Tris-base and 10 mM EDTA, pH 8.3–4 °C; Doumit & Koohmaraie, 1999) with addition of 0.1% of 2-mercaptoethanol and 2% of protease cocktail inhibitor (P8340, Sigma, St. Louis, MO). The homogenization process followed the description above, except that the last centrifugation was at 20,000 ×*g* for 30 min. The supernatant was transferred to a new tube and represents the sarcoplasmic fraction that was used for analysis. Protein concentration and final sample preparation were performed as described above.

All other proteins analyzed (calpain-1, procaspase-3, HSP70, gelsolin, alpha-II-spectrin, desmin and troponin-T) were extracted using same procedure and preparation as described in Wright et al. (2018) and kept in −20 °C until loaded in gels.

2.5. SDS-PAGE

Acrylamide resolving gels (20 cm wide x 8.5 cm tall x 0.75 mm thick) with 5% acrylamide for stacking gel were used. Different resolving gels concentrations were prepared according to protein size (alpha-II-spectrin, calpain-1 and calpastatin: 7% with 25, 15 and 15 μg protein/well, respectively; desmin, troponin-T, procaspase-3, gelsolin and HSP70: 10%, with 10, 5, 20, 15 and 20 μg protein/well, respectively; ATP5A and COX4 15%, 15 μg of protein/well) and the running procedure used an MGV-202-20 electrophoresis unit (C.B.S. Scientific, San Diego, CA) with an initial running at 60 V for 20 min in running buffer (25 mM tris-base, 0.2 M glycine and 0.1% SDS) followed by an additional running at 125 V with time adjusted to each protein.

2.6. Immunodetection

Immediately after electrophoresis, proteins were transferred from gels to a nitrocellulose membrane (Thermo Scientific, Rockford, IL)

using a wet tank system (EBU-402, C.B.S. Scientific, San Diego, CA) with cold transfer buffer (50 mM tris-base, 0.38 M glycine, 10% SDS and 10% methanol). Proteins were transferred at 500 mA for 1 h and membranes were dried overnight. Membranes were stained using total protein stain protocol (REVERT™, LI-COR, Lincoln, NE), and signal from the total protein stain was used to normalize target protein signal. All membranes were briefly (2 min) washed after total protein stain protocol with 1× TBS (20 mM Tris-base, 140 mM NaCl, pH 7.6) and then blocked with StartBlocking™ (TBS) Blocking Buffer (Thermo Scientific, Rockford, IL) for 1 h, followed by primary antibodies incubation diluted in blocking buffer with 0.2% tween-20. Anti-ATP5A (ab14748, Abcam, Cambridge, MA) and anti-COX4 (4850, Cell Signaling, Danvers, MA) were diluted 1: 1000; anti-calpain-1 (MA3–940, Thermo Scientific, Rockford, IL) diluted 1: 10,000 and anti-calpastatin (MA3–944, Thermo Scientific, Rockford, IL) diluted 1: 5000; anti-alpha-II-spectrin (SC-48382, Santa Cruz, Dallas, TX) diluted 1: 250; anti-desmin (D1033, Sigma, St. Louis, MO) diluted 1: 10,000 and anti-troponin-T (T6277, Sigma, St. Louis, MO) diluted 1: 20,000; anti-procaspase-3 (9668, Cell Signaling, Danvers, MA) diluted 1: 1000, ant-gelsolin (12,953, Cell Signaling, Danvers, MA) diluted 1: 1000 and anti-HSP70 (4872, Cell Signaling, Danvers, MA) diluted 1: 1000 were used. After primary incubation, membranes were washed four times with 1× TBS-0.1% tween-20 for 5 min. Secondary antibody conjugated with near-infrared (IRDye 800CW, LI-COR, Lincoln, NE) was selected based on host from primaries, and diluted in same solution as primaries. After secondary incubation, membranes were washed as above and followed with 1× TBS pH 7.6. Membranes were scanned using Odyssey CLx (LI-COR, Lincoln, NE). Bands were quantified using Image Studio software version 5.2.

Calpain-1 autolysis was calculated according to formula: 76 kDa band / sum of signal of total bands (80 + 78 + 76 kDa). Desmin and troponin-T degradation reported as formula = signal of intact band / sum of signal of total bands (intact + degradation products). Alpha-II-spectrin was identified by three bands: first one with molecular weight > 250 kDa, representing the intact protein and the two lower bands (150 and 145 kDa), products of degradation. Appearance of 150 kDa band was investigated and related to total signal, using formula: alpha-II-spectrin (150 kDa) = 150 kDa band / sum of signal of total bands (250 + 150 + 145 kDa).

2.7. Statistical analysis

Data were analyzed using a complete randomized block design with slaughter dates as a blocking factor (May 24th, June 28th and August 2nd) and animal as a random effect. The model to describe parameters that were measured once (i.e. protein content: ATP5A, COX4, procaspase-3, HSP70, gelsolin, ratio of calpastatin at 24 and 1 h; and enzymes activities: LDH and CS) only tested the fixed effect of biological type (Angus, Brangus and Brahman). The model to investigate parameters that were measured at three times *postmortem* included time as a fixed effect and the interaction effect of biological type and time *postmortem* (i.e. ATP and calpastatin). The model used to describe the parameters that were measured over time (more than three times *postmortem*) considered the best fit for each one in a regression curve. As described by Bruce, Scott, and Thompson (2001), an exponential decay curve was tested to describe pH decline *postmortem* and it was a good fit for the three biological types tested. Therefore, predicted means were compared using *t*-test for pH decline, as well as for carcass temperature, calpain-1 autolysis, troponin-T degradation and alpha-II-spectrin band 150 kDa formation. The model used to describe desmin degradation, however, included a two-step approach in which Angus and Brangus were modeled as exponential and Brahman as linear decay. Models were combined and predicted means for biological types compared by two-sample t-test.

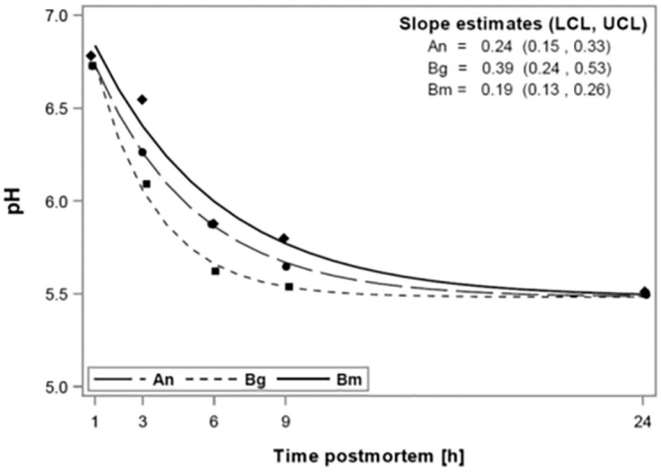

Fig. 1. Exponential curve representing pH decline in *Longissimus lumborum* from three cattle biological types during 24 h *postmortem*. An = Angus; Bg = Brangus; Bm = Brahman.

3. Results and discussion

3.1. Muscle pH and postmortem metabolism

Exponential decay slope estimates for LL pH decline were similar (P = 0.43) between Angus and Brahman, and both were different (P = 0.02) from Brangus. Angus showed an intermediate pH decline (Fig. 1). Brahman exhibited the lowest slope estimate (b = 0.19), indicating resistance to pH decline. Predicted means comparison revealed that pH decline at 3 h *postmortem* was different (P < 0.05) between biological types and Brahman LL showed higher mean (predicted pH 3 h = 6.40) compared to Angus and Brangus (6.25 and 6.06, respectively). At 6 h *postmortem*, those differences were reduced (P = 0.08) between Angus and Brahman but remained significant (P < 0.05) between Brangus compared to Angus and Brahman. This pattern was maintained at 9 h *postmortem*. At 24 h *postmortem*, predicted means were similar (P > 0.05) between biological types. Exponential decay slope estimates for LL temperature were similar (P > 0.05) between biological types.

The resistance to pH decline evidenced by Brahman carcasses agrees with the notion that carcasses with high or low pH at 3 h *postmortem* may produce beef with less acceptable tenderness compared to beef that has a moderate pH (i.e. pH 6.3) at this time, as reviewed by Huff-Lonergan, Zhang, and Lonergan (2010). Preservation of muscle pH closer to physiological conditions suggests greater capacity to protect cellular ATP levels. To address this possibility, muscle ATP levels were determined at 1, 3, and 6 h *postmortem* (Fig. 2). Biological type tended to influence ATP (P = 0.10), with Brahman exhibiting numerically higher ATP compared to Angus, regardless of time. The pattern of ATP

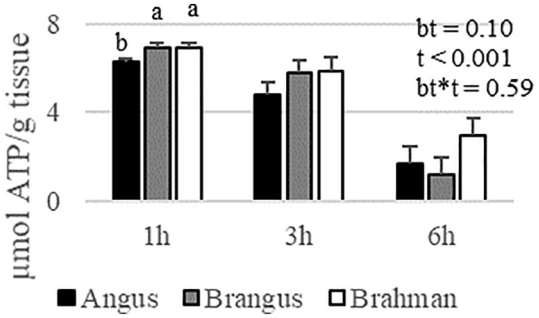

Fig. 2. ATP concentration in *Longissimus lumborum* from three cattle biological types during 6 h *postmortem*. Slice effect 1 h: P = 0.05. Means ± standard error.

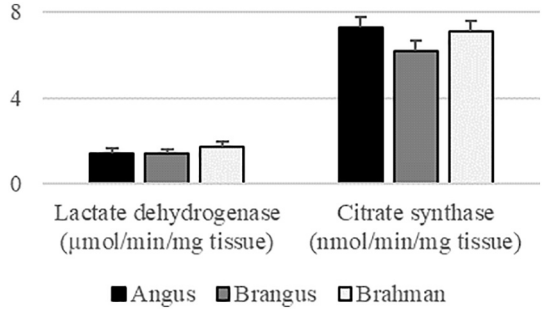

Fig. 3. Activities of lactate dehydrogenase (P = 0.48) and citrate synthase (P = 0.23) in *Longissimus lumborum* from three cattle biological types. Means ± standard error.

decline over time was not different between biological types (biological type x time *postmortem*, P = 0.59); however, 1 h slice effect (P = 0.05) revealed that Brahman muscle contained higher (P < 0.05) ATP than Angus. Interestingly, ATP content in Brahman LL at 6 h was 1.8 times higher than in Angus.

Greater ATP at 1 h in Brahman LL may be due to greater ATP generation, decreased ATP hydrolysis, or a combination. As suggested before, ATP production from aerobic metabolism of glycogen is ~10-fold greater than anaerobic glycolysis, which means that even a small increment in oxidative phosphorylation would represent a considerable boost in ATP production (Scheffler et al., 2015). Therefore, enzymatic activities of LDH and CS were measured as indicators of glycolytic and oxidative metabolism, respectively. Biological types have similar (P = 0.48) LDH and CS (P = 0.23) activities in LL 1 h *postmortem* (Fig. 3). As CS activity is often used as a marker of mitochondrial content, this indicates that quantity of mitochondria is similar between groups. However, we previously observed that CS activity was positively associated with increasing proportion of Brahman influence (Wright et al., 2018). Since enzyme activity can be related to quantitative and/or qualitative changes in the protein, we chose to further investigate mitochondrial content in the LL. The protein expression of two mitochondrial proteins involved in oxidative phosphorylation were evaluated by Western blotting: ATP synthase subunit alpha (ATP5A) and cytochrome *c* oxidase subunit 4 (COX4). Total protein content and profile were similar between biological types (Fig. 4A). No differences were found between biological types regarding content of mitochondrial proteins ATPA5 (P = 0.51), COX4 (P = 0.49; Fig. 4B and C).

Even though mitochondria protein content may be similar, mitochondrial function may differ between biological types and influence energy levels. Alternatively, the mitochondrial ATPase may function

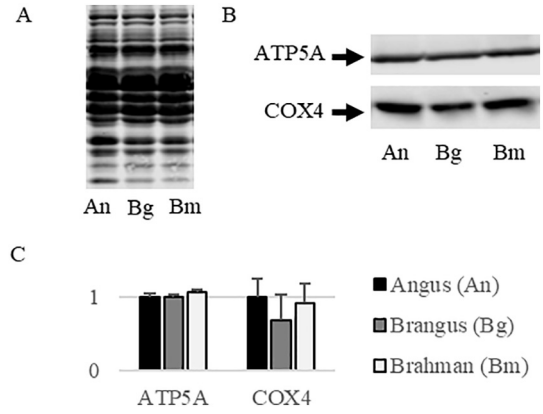

Fig. 4. A) Total protein stain; B) Immunodetection of mitochondrial proteins ATP5A and COX4; C) Content of ATP5A (P = 0.51) and COX4 (P = 0.49) in *Longissimus lumborum* from three cattle biological types. Means ± standard error.

backward *postmortem*, thereby contributing to ATP hydrolysis rather than ATP production (Hudson, 2012). Thus, there may be functional differences in Brahman compared to Angus that relate to lower ATP turnover *postmortem*. There is limited evidence to support that differences in function relate solely to shifts in protein expression in various biological types (Rodrigues et al., 2017). Instead, regulation of pathways by metabolites and post-translational modifications may be more decisive in determining muscle metabolism between biological types.

In *postmortem* muscle, declining ATP levels can influence *rigor mortis* shortening, as ATP is needed to release myosin from actin. On the other hand, ATP levels differentially influence calcium uptake by sarcoplasmic reticulum and mitochondria; although both exhibit maximal uptake at 3 mM, mitochondria calcium uptake is more affected by ATP than sarcoplasmic reticulum (Whiting, 1980). Moreover, Whiting (1980) also showed that mitochondria begin to lose their ability to sequester calcium near pH 6.5, whereas sarcoplasmic reticulum calcium uptake capacity begins to decrease near pH 6.0. Depletion of creatine phosphate and reduced ATP levels precede rapid phase of shortening, which starts when pH is between 6.6 and 6.3, suggesting that calcium release by mitochondria is more important for rigor shortening than by sarcoplasmic reticulum (Hertzman, Olsson, & Tornberg, 1993). Interestingly, in the present study, predicted mean pH at 3 h *postmortem* ranged from 6.06 to 6.40, but only the predicted mean pH of Brahman LL was higher than 6.30 at this time, indicating that calcium may be trapped in the mitochondria matrix in Brahman. Lower sarcoplasmic calcium availability directly affects calpain-1 activation (Goll et al., 2003).

Release of calcium to the sarcoplasm will contribute to calpain activation and consequently, proteolysis. Decreasing oxygen availability impairs mitochondria respiration and limits ATP production; increasing levels of lactate and hydrogen ions from anaerobic glycolysis contributes to carcass pH decline. Comparison between rapid (pH 3 h: 5.9–6.1) and slow (pH 3 h: 6.6–6.9) *Longissimus dorsi* acidification rate showed panelists rated 2 and 6d aged beef from the rapid pH decline group as more tender (O'Halloran, Troy, Buckley, & Reville, 1997). Additionally, there is an ideal rate in which glycolysis can positively influence tenderization (Hwang & Thompson, 2001).

3.2. Calpain-1 autolysis, calpastatin content and proteolysis

Exponential slope estimates representing calpain-1 autolysis and activation during *postmortem* were different (P < 0.05) between biological types (Fig. 5A). This model represents the pattern of band formation observed in the immunoblots (Fig. 5B). Predicted means were similar (P > 0.05) between biological types during 1 and 3 h *postmortem*. At 6 h, Brangus LL predicted means were greater (P < 0.05) than Angus and Brahman. After 24 h, predicted means were different (P < 0.001) between biological types, with Brangus LL showing greater calpain-1 autolysis, followed by Angus and lower for Brahman compared to both. At 7d *postmortem*, LL calpain-1 autolysis was similar (P = 0.96) between Angus and Brangus, but both were different (P = 0.002) than Brahman. After 14d *postmortem*, predicted means for calpain-1 autolysis were similar (P > 0.05) between biological types. Interestingly, calpain-1 autolysis followed the same pattern as pH decline between biological types. Brangus LL showed faster rate of pH decline and faster rate of calpain-1 autolysis. On the other hand, Brahman showed resistance to pH decline and slower rate of calpain-1 autolysis. Angus is intermediate for both parameters and it is related with greater tenderization.

The progression of calpain-1 autolysis can be reduced by calpastatin (Koohmaraie, 1992). Immunoblots for calpastatin showed a range of bands with molecular weight varying from 130 kDa (intact calpastatin) to approximately 30 kDa bands (products of degradation), which agrees with previous results (Doumit & Koohmaraie, 1999). Calpastatin breakdown pattern showed slight differences among biological types (Fig. 6A) that did not represent statistical differences after bands were

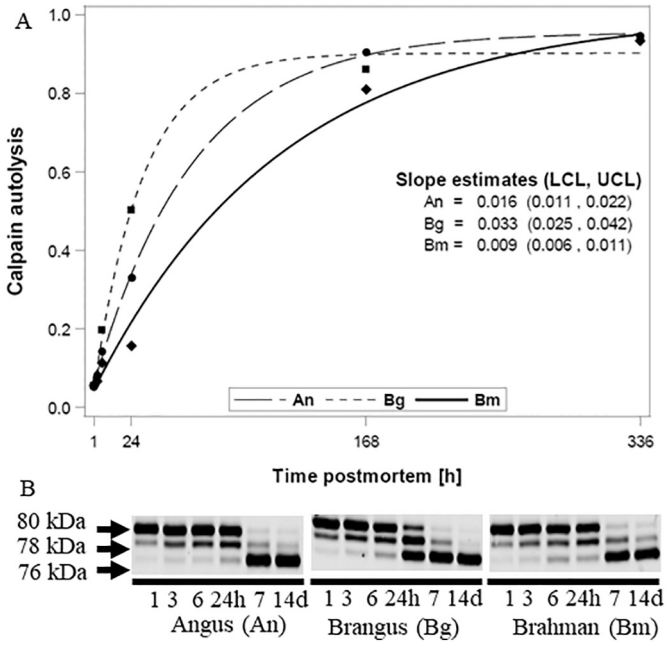

Fig. 5. A) Exponential curve representing calpain-1 autolysis (formation of 76 kDa band/total signal); B) immunodetection of calpain-1 in *Longissimus lumborum* from three biological types during 14d *postmortem* aging.

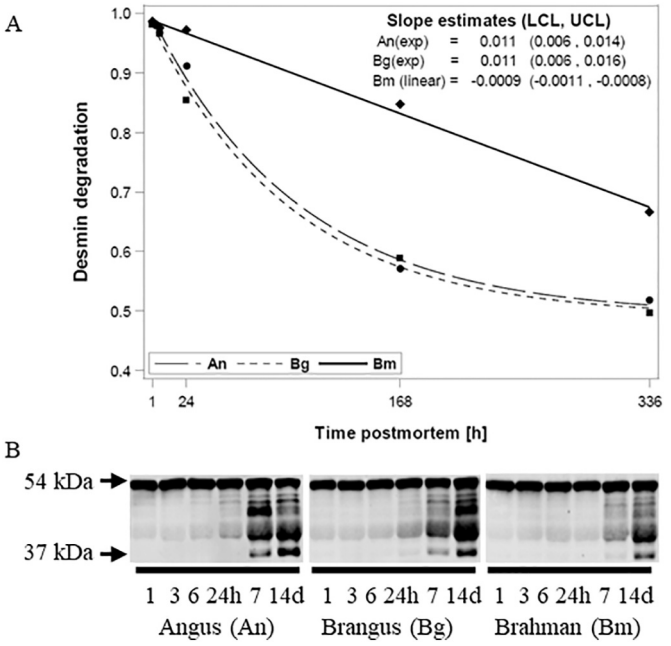

Fig. 7. Exponential curve representing desmin degradation (intact band/total signal); B) immunodetection of desmin in *Longissimus lumborum* from three biological types during 14d *postmortem* aging.

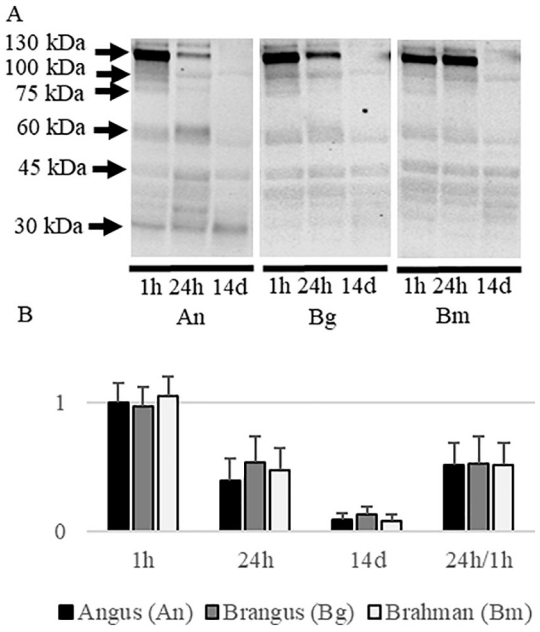

Fig. 6. A) Immunodetection of calpastatin; B) calpastatin content in *Longissimus lumborum* from three cattle biological types during 14d *postmortem* aging. Means ± standard error.

quantified (Fig. 6B). The absence of those differences is probably due to relatively large variation between individuals within the same biological type. This variation is aligned with high standard deviation values found for WBSF even after 14d aging, mostly related with steaks from Brahman (11 N). Calpain is inhibited by calpastatin at lower calcium concentrations than that required for calpain activity. Therefore, calpastatin-mediated inhibition of calpain could happen earlier or be sustained longer in Brahman. The reduction of calpain-1 autolysis should consequently decrease myofibrillar proteolysis.

Desmin degradation pattern was better represented by exponential decay for Angus and Brangus and linear decay for Brahman (Fig. 7A). Predicted means at 24 h, and 7 and 14d were higher ($P > 0.05$) for Brahman LL compared to Angus and Brangus. In agreement, persistence of the intact band (54 kDa) and slower formation of degradation bands during aging (Fig. 7B) in Brahman LL represented less extensive desmin degradation. It is possible that muscle structure and mitochondria morphology are preserved longer in Brahman LL. Desmin is an intermediate filament responsible for organizing muscle structure in vivo and holding mitochondria, and its degradation is potentially related with mitochondria disruption. In this case, Angus and Brangus LL showed greater desmin degradation earlier which agrees with positive correlations between mitochondrial proteins release during aging, calpain-1 activation and improved tenderness between three cattle breeds and two muscles with different metabolism (Gagaoua et al., 2015).

Exponential decay that represents troponin-T degradation was influenced by biological types (Fig. 8A). Slope estimates were different ($P = 0.003$) between Brahman and Brangus. Predicted means were different ($P < 0.05$) between Brahman and other biological types starting at 24 h and maintained throughout 14d. Consistent with desmin degradation, Brahman showed delayed troponin-T degradation, and importantly, did not reach the same extent of degradation by the end of the 14d aging period. Troponin-T degradation is consistent with the aforementioned observations and is related with faster pH decline. Angus showed intermediate slope estimate and Brahman the lowest, meaning less degradation. Results reinforced that the appearance of 28 kDa band during *postmortem* storage (Fig. 8B) is the most noticeable and reported change associated with tenderization (Koohmaraie, 1994).

Calpain-1 autolysis, calpastatin, and proteolysis of desmin and troponin-T are consistent with slower and less extensive tenderization in Brahman LL. Calcium dependent systems, calpain and calpastatin, were differentially affected among biological types and resulted in reduced proteolysis. Although markers of mitochondria content were not different between biological types, mitochondria may play important roles in postmortem muscle by affecting calcium uptake from the sarcoplasm, altering energy status early *postmortem,* or by triggering cell death pathways that impact proteolysis.

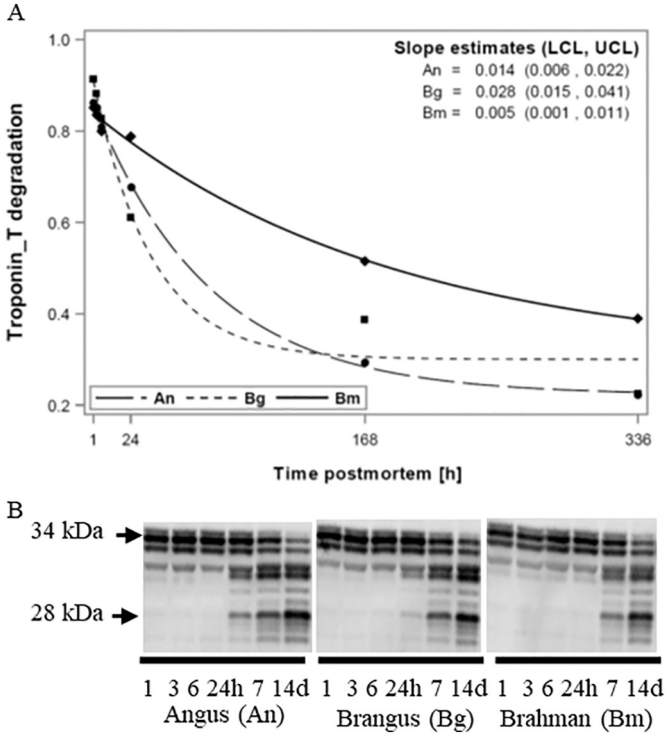

Fig. 8. Exponential curve representing troponin-T degradation (intact band/total signal); B) immunodetection of troponin-T in *Longissimus lumborum* from three biological types during 14d *postmortem* aging.

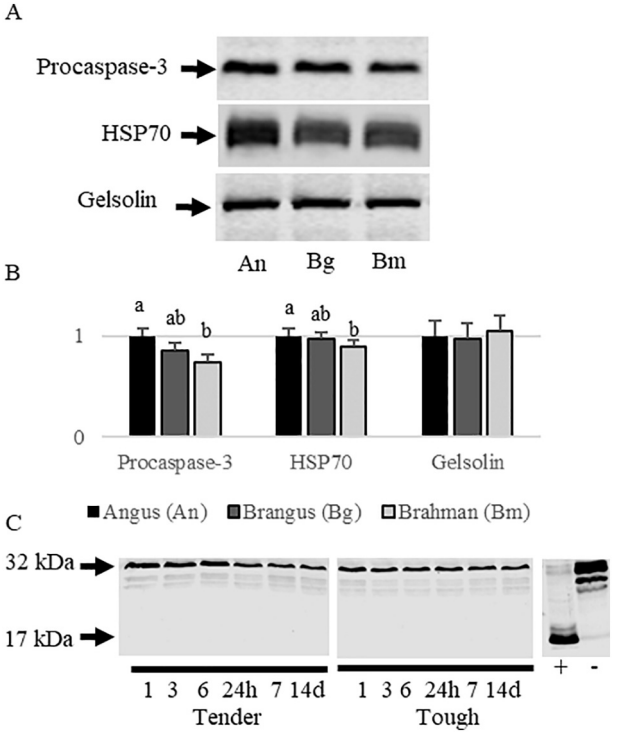

Fig. 9. A) Immunodetection of procaspase-3, heat shock protein 70 (HSP70) and gelsolin; B) Content of procaspase-3 ($P = 0.02$), HSP70 ($P = 0.02$) and gelsolin ($P = 0.90$); in *Longissimus lumborum* from three cattle biological types. Means ± standard error with different letters are different ($P < 0.05$); C) tender sample is from Angus with Warner-Bratzler shear force at 14d of 34.4 N and sensory tenderness of 5.6 in a 1 to 8 scale, being 1 very tough and 8 very tender; tough sample is from Brahman with 57.0 N and 3.0, instrumental and sensory tenderness, respectively; positive (+) and negative (−) controls for cleaved caspase-3 were loaded as reference.

3.3. Mitochondrial-mediated cell death via caspase-3 system

Caspases are involved in mitochondrial-mediated cell death, and they have been proposed to mediate tenderization via their role in calpastatin degradation (Kemp, King, Shackelford, Wheeler, & Koohmaraie, 2009; Wang et al., 1998). Caspase activation early *postmortem* may be related to biological type; Brahman muscles show resistance to pH and ATP decline, which could delay caspase mediated cell death and result in slower degradation of calpastatin. Caspase-3 is an executioner of apoptosis and initiates protein degradation in muscle in catabolic conditions (Du et al., 2004); caspase-3 has also been implicated in *postmortem* muscle proteolysis (Kemp et al., 2006; Kemp & Parr, 2008). The zymogen procaspase-3 (32 kDa) must be cleaved for activity, resulting in fragments of approximately 17 kDa and 12 kDa (Nicholson et al., 1995). Immunoblotting for intact and cleaved caspase revealed only one band with molecular weight around 32 kDa (Fig. 9A). Content of procaspase-3 differs ($P = 0.02$) among biological types; Angus LL showed greater content than Brahman, and Brangus showed intermediate content (Fig. 9B). If procaspase-3 is cleaved and activated *postmortem*, higher initial procaspase-3 content for Angus LL could lead to greater caspase-3 activity. However, attempts to detect cleaved caspase-3 in *postmortem* muscle were not successful; this included increasing protein loading and evaluating intact and cleaved caspase-3 from 1 h to 14d in individuals with large differences in instrumental and sensory tenderness (Fig. 9C). If cleaved caspase-3 is being generated but cannot be detected, one would expect that content of procaspase-3 would decrease *postmortem*. However, it was not the case and procaspase-3 content remained similar throughout 14-d period for all biological types.

Caspase-3 activation in skeletal muscle *postmortem* is controversial. On one hand, there are reports showing cleaved and consequent active caspase-3 during *postmortem* (Cao et al., 2013; Chen, Feng, Zhang, Xu, & Zhou, 2012; Huang, Huang, Xue, Xu, & Zhou, 2011). However, others did not detect the 17 kDa fragment of cleaved and active caspase-3 (Cramer, Penick, Waddell, Bidwell, & Kim, 2018; Mohrhauser, Underwood, & Weaver, 2011; Underwood, Means, & Du, 2008). Even though the cleaved band was not detected in our study, higher procaspase-3 content in Angus LL is in agreement with evidence that more tender beef has greater caspase-3 gene expression (Bernard et al., 2007). Additionally, an in vitro study showed that active caspase-3 is related with calpastatin cleavage at early times *postmortem* (Huang et al., 2014), but the resulting pattern of calpastatin fragments is different than bands visualized in our study.

Since cleaved caspase-3 could not be detected, apoptotic factors upstream and downstream of caspase-3 activation were considered. Heat shock protein 70 (HSP70), a chaperone with anti-apoptotic effects, has been shown to prevent cytochrome *c* release from mitochondria and block formation of the apoptosome upstream of caspase-3 activation (Saleh, Srinivasula, Balkir, Robbins, & Alnemri, 2000). Although biological type impacted HSP70 ($P = 0.02$), Angus exhibited greater ($P < 0.05$) HSP70 than Brahman (Fig. 9A and B). Thus, the pattern was not consistent with the idea of apoptosis being favored in Angus muscle. Another anti-apoptotic protein, gelsolin, was also evaluated. Previously, Koya et al. (2000) showed that gelsolin blocks cytochrome *c* release and caspase-3 activation in cells in the presence of apoptosis-stimulating agents. However, gelsolin content was not different ($P = 0.90$) between biological types (Fig. 9A and B). For downstream processes, activated caspase-3 can cleave certain proteins; therefore, proteolysis of caspase-3 targets provides evidence of caspase-3 activity and apoptosis. Alpha-II-spectrin is a calpain-1 and caspase-3 substrate, with degradation resulting in different pattern of fragments, depending on protease. Both protease systems contribute to the formation of a 150 kDa band. While calpain activity results in an additional 145 kDa fragment, caspase-3 activity results in cleavage of the initial 150 kDa

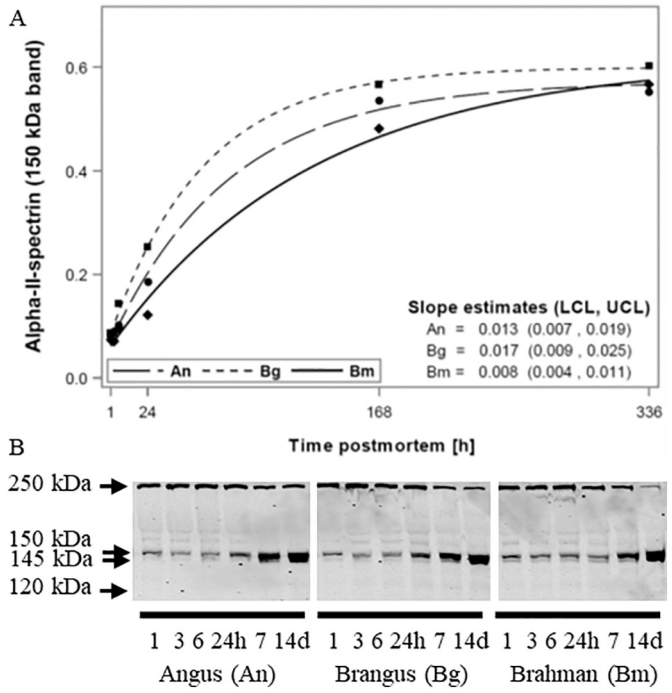

Fig. 10. Exponential curve representing formation of alpha-II-spectrin 150 kDa band (150 kDa band/total signal); B) immunodetection of alpha-II-spectrin in *Longissimus lumborum* from three biological types during 14d *postmortem* aging.

fragment to a 120 kDa fragment (McGinnis, Gnegy, Park, Mukerjee, & Wang, 1999). Exponential model representing formation of the 150 kDa band was influenced by biological types (Fig. 10A). Predicted means were similar ($P > 0.05$) between biological types at 1 and 3 h *postmortem*. After 6 h, Brangus LL showed greater ($P = 0.02$) band formation than Brahman LL, but similar ($P = 0.19$) to Angus. At 24 h *postmortem*, Angus and Brangus predicted means were different ($P < 0.05$) than Brahman. At 7d, difference ($P < 0.001$) was only detected between Brangus and Brahman. By 14d, no differences ($P > 0.05$) remained. Once more, results for alpha-II-spectrin were aligned with faster pH decline and early calpain-1 autolysis observed in Brangus. Degradation of alpha-II-spectrin showed bands with molecular weights of 150 and 145 kDa (Fig. 10B).

Alpha-II-spectrin degradation in beef was reported previously (Saccà, Pizzutti, Corazzin, Lippe, & Piasentier, 2016). In this case, the authors identified a 120 kDa band early *postmortem* (20 min after slaughter), and it was not detected after 48 h; and the intact band (250 kDa) was not present at 7d. Similarly, mechanical ventilation induced proteolysis of diaphragm in rats generated both calpain and caspase spectrin breakdown products (145 and 120 kDa, respectively) (Smuder et al., 2018). Conversely, (Meary et al., 2007) incubated liver at 4 °C for 48 h to examine the pattern of spectrin breakdown products. In this case, the profile mirrored calpain digestion: alpha-II-spectrin was completely cleaved resulting in one band at 145 kDa, but the 120 kDa band was not generated. In our experiment, the caspase-specific spectrin breakdown product (120 kDa) was not detected either, and some intact alpha-II-spectrin (250 kDa) remained at 14d *postmortem*. Lack of a caspase specific spectrin breakdown product corroborates the absence of cleaved caspase-3 fragment mentioned earlier. Altogether, the proteolytic fragments support that proteolysis *postmortem* is greatly influenced by calpain-1 rather than caspase-3.

4. Conclusions

Resistance to LL pH decline within first 9 h, greater ATP levels at 1 h, and delayed calpain-1 autolysis confirmed our hypothesis that Brahman LL has greater resistance to cellular stresses *postmortem*. This resistance does not seem to be related to glycolytic capacity (i.e., similar LDH activity) but is impacted by early *postmortem* differences in energy status. Additionally, Brahman LL has reduced procaspase-3 content, but after further investigation, it was shown that mitochondria-mediated apoptosis and proteolysis by caspase-3 is unlikely. There is no evidence of caspase-3 cleavage *postmortem*, and upstream and downstream apoptotic factors are consistent with this. Although mitochondria-mediated apoptosis is not likely, other aspects of mitochondria function may be important. Mitochondria roles in energy production and calcium sequestration may contribute to delayed calpain-1 activation, thereby decreasing myofibrillar proteolysis and negatively impacting tenderization in indicine cattle. Further work is necessary to confirm the roles of mitochondrial functions in postmortem metabolism and tenderization.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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